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The DNA binding domain of the Varicella-zoster virus gene 62 protein interacts with multiple sequences which are similar to the binding site of the related protein of herpes simplex virus type 1

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ABSTRACT

Varicella-zoster virus gene 62 encodes a protein with predicted Mr of 140,000D (VZV 140k) that shares extensive predicted amino acid sequence homology with the major immediate early (IE) transcriptional regulator protein of herpes simplex virus type 1 (HSV-1) Vmw175. The integrity of highly conserved region 2 is essential for the DNA binding and transcriptional regulatory functions of Vmw175. Similarly, an insertion mutation in region 2 (codons 468–641) of 140k eliminates the transcriptional repression and activation functions of this protein. We have expressed a fragment of 140k which encompasses region 2 as a non-fusion polypeptide in bacteria. This 140k DNA binding domain peptide (codons 417–646) binds to numerous DNA sequences throughout the VZV gene 62 promoter region. It induces multiple regions of protection from DNase I digestion, flanked by sites of DNase I hypersensitivity. Several of the sites recognized can be considered to be divergent forms of the consensus sequence which is recognized by Vmw175. However, by use of a panel of mutagenized probe fragments, we found that the 140k DNA binding domain was less sequence-specific than Vmw175 in its interactions with DNA. Consistent with this, the homologous Vmw175 DNA binding domain, and also intact Vmw175, recognize the gene 62 binding sites much less efficiently than the 140k DNA binding domain. Also in contrast to the situation with Vmw175, the140k DNA binding domain failed to induce DNA bending when occupying the binding sites in its own promoter. Deletion analysis has mapped the minimal DNA binding domain of the VZV 140k protein, as measured in gel retardation analysis, to lie within residues 472 to 633. The differences in binding characteristics of the DNA binding domains of the homologous VZV 140k and HSV-1 Vmw175 IE proteins may account for the subtle differences in their regulatory activities in transfection assays and during virus growth in tissue culture.

INTRODUCTION

Expression of many herpesvirus genes is coordinately regulated and sequentially ordered in a cascade fashion. Gene regulation during herpes simplex type 1 (HSV-1) infection in tissue culture has been most extensively analyzed. HSV-1 genes are classified as immediate-early (IE), early or late, depending on their kinetics of expression and response to inhibitors of macromolecular synthesis (1,2). The five IE genes are transcribed at the onset of infection in the absence of de-novo protein synthesis and at least some of the IE polypeptides are required for the normal regulation of the later classes of genes (for reviews see 3–5). Of the five IE gene products, Vmw175 (expressed from gene IE-3) is perhaps the most important since its inactivation results in failure to transcribe early and late genes and an apparent over-expression of the IE genes (6,7). Thus Vmw175 is directly or indirectly required for the normal expression of all other HSV-1 genes.

Varicella-zoster virus (VZV), another member of the neurotropic alphaherpesvirinae subfamily, is genetically closely related to HSV-1 (8,9). However, in contrast to HSV-1, the knowledge of gene regulation during VZV infection is still rudimentary. The major reason for this is the poor growth of VZV in the available tissue culture systems which produce predominantly cell associated virus with low titer stocks. Four VZV IE products have been detected in infected cells by cycloheximide reversal experiments (10). The gene product of VZV genes 62 and 71 (the identical gene is present in both copies of the short repeat regions (Figure 1A)) has a predicted Mr of 140,000D (VZV 140k) and shares considerable predicted amino acid identity with HSV-1 Vmw175. For several reasons 140k is believed to be the functional counterpart of Vmw175; HSV-1 mutants with lesions in Vmw175 can be complemented by either transfected plasmids or transformed cell lines which express VZV 140k (11,12) and moreover a recombinant HSV-1 virus (HSV-140) with both copies of the Vmw175 coding sequences replaced by the homologous gene 71 ORF is viable in tissue culture (13).

In transfection assays at least, the VZV 140k protein functions as a powerful transcriptional activator of viral and selected cellular gene expression (14–16) and autoregulates its own gene 62

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promoter (17). The predicted primary sequence of 140k can be divided into five regions on the basis of similarity to the corresponding Vmw175 protein (18). The integrity of highly conserved region 2 is required for the transactivation and repression phenotypes of Vmw175 (19,20) and an insertion mutation in 140k region 2 inhibits these same functions (17) suggesting that VZV 140k and HSV-1 Vmw175 regulate gene expression by related mechanisms. However the two proteins are not entirely interchangeable for autoregulation as Vmw175 can substitute for 140k in the downregulation of the VZV gene 62 promoter in transient transfection assays whereas the 140k protein does not repress the HSV-1 IE-3 promoter in the reciprocal experiment (17).

In the case of Vmw175, its autoregulatory function is mediated at least in part by the formation of a complex between Vmw175 and a specific sequence fitting the Vmw175 binding consensus ATCGTnnnnnYSG (21,22 and references therein) found at the cap site of the IE-3 promoter (23). This complex is presumed to downregulate transcription either by sterically blocking the movement of RNA polymerase II or by displacing an essential transcriptional factor such as TFIID. The DNA binding domain of Vmw175 has been mapped by a variety of different techniques to a region encompassing the C-terminal protion of region 1 and the whole of region 2 (19,20,24,25). This domain of Vmw175 has been expressed as a stable, functionally separable domain in bacteria (25,21), exists as a dimer in solution (21) and binds DNA with a specificity similar to that of the intact protein (25,21). Both intact Vmw175 and its isolated DNA binding domain bend DNA when occupying the IE-3 promoter binding site (26). The biological significance of this DNA bending is unclear but it may be relevant to the mechanism of autoregulation by Vmw175.

The corresponding DNA binding domain of VZV 140k has been expressed as a trpE fusion polypeptide in bacteria (encompassing codons 417 to 647) (27) and binds to sequences in the vicinities of both cap sites proposed for its own gene 62 promoter (28,12). Degenerate versions of the HSV-1 Vmw175 binding consensus are contained within these recognized sequences. In addition, the same group demonstrated that the DNA binding domain fusion polypeptide of the homologous IE180 protein of pseudorabies virus (PRV) interacted with its promoter in a similar manner (27). These findings suggest that the formation of a specific complex between an IE protein and its own gene promoter may be a common mechanism used by alphaherpesvirinae to autoregulate transcription of an essential IE gene.

We sought to investigate whether differences in the behaviour of 140k and Vmw175 in autoregulation assays could be explained by variations in their DNA binding specificities. We have expressed the region 2 domain of 140k as a non-fusion protein in *E.coli* (codons 417 to 646) and analyzed its activities in DNA binding experiments in comparison with the corresponding Vmw175 DNA binding domain. Our results confirm those of Wu and Wilcox (27) and extend them to identify in more detail the sites that are bound by the VZV 140k DNA binding domain. A mutational analysis indicated that the VZV 140k DNA binding domain was less specific in its DNA binding requirements than that of Vmw175, and failed to bend DNA at its binding sites. These differences in DNA binding properties may be relevant to the slight differences in their biological properties. In addition, we have defined the limits of the VZV 140k DNA binding domain to residues 472–633 using a deletion analysis. This is significantly smaller than the minimal DNA binding domain of HSV-1 Vmw175 (21,25).

MATERIALS AND METHODS

Plasmids, bacteria and bacteriophage

Plasmid pV17 is a derivative of p140 (a plasmid including the entire VZV gene 62, see ref 28) with a 12 bp EcoRI linker oligonucleotide inserted at codon 418. Plasmid p585T7a was derived from the T7 expression vector p585.4 (21) by removal of the EcoRI site (by cutting, Klenow filling-in of 5′ overhangs followed by religation) prior to co-ligation of an NcoI-EcoRI adaptor oligonucleotide (21) and the EcoRI-HindIII multiple cloning region (mcr) fragment of PUC19 between the NcoI and HindIII sites of p585.4 at the initiation of translation. In addition, the SphI-PvuII fragment of the vector was removed by cutting with SphI and PvuII, Klenow end-filling, followed by blunt-end ligation, to increase the number of restriction enzyme sites available for cloning in the p585T7a vector. All plasmids were constructed and maintained in *E.coli* strain ΔH5. For the expression experiments, plasmids were transformed into *E.coli* strain BL21 (DE3) pLysS (29).

Bacteriophage M13 containing wild type and mutant forms of the HSV-1 IE-3 cap site region (21) were transfected into *E.coli* strain JM101. The JM101 host bacterium was used for all subsequent growth and maintenance of M13 bacteriophage.

Preparation of intact Vmw175 protein

Crude nuclear extracts containing HSV-1 Vmw175 protein were prepared from HeLa cells transfected with the plasmid p175 as previously described (30).

Construction of the 140k DNA binding domain expression plasmids

Plasmid p585T7aVT2 was constructed by cutting the plasmid pV17 with BstEII, filling-in of the 5′ overhang with Klenow, before cutting again with EcoRI. The resulting EcoRI-BstEII fragment (containing the 140k codons 417 to 646) was isolated and inserted between the adaptor EcoRI site and the SmaI site of the vector p585T7a. The NcoI-EcoRI adaptor oligonucleotides had been synthesized so as to maintain the 140k peptide reading frame (which is initiated at the ATG of the NcoI site). BamHI digestion, followed by isolation and religation of the plasmid construct removed DNA sequences between the BamHI site of the mcr and the BamHI site in the translational termination region. The sequences at the 5′ and 3′ ends of the insertion were confirmed by direct plasmid sequencing. Plasmid p585T7aVT2 encodes a 257 residue peptide, designated VT2, consisting of 4 amino acids derived from the amino-terminal junction region sequence (MAEF); the VZV 140k residues 417 to 646; 3 residues from the mcr (QGI) to its carboxy-terminal side; followed by 20 residues encoded by vector DNA 3′ to the BamHI site. Plasmid p585T7aVT2X was derived from p585T7aVT2 by insertion of an XbaI linker oligonucleotide containing stop codons in all three reading frames into the BamHI site of p585T7aVT2—this removed 19 vector encoded residues from the distal end of the expressed protein that are present in the VT2 polypeptide. The 238 residue expression product of plasmid p585T7aVT2X is designated VT2X.

Plasmids expressing 140k DNA binding domain deletion polypeptides were constructed using appropriate restriction enzyme sites (as indicated in Figure 8A) for cloning into the mcr of plasmid p585T7a. The cloning proceedures ensured that all constructs were in frame with the ATG translation initiation codon of the vector. The predicted 5′ and 3′ boundaries of the expression regions were confirmed by direct plasmid DNA sequencing.

Expression and partial purification of the 140k DNA binding domains

T7 expression plasmids were transformed into *E.coli* host strain BL21 (DE3) pLysS and maintained as glycerol stocks. Bacterial colonies from freshly streaked plates containing ampicillin at 100μg/ml and chloramphenicol at 25μg/ml were innoculated into YT-broth containing the above concentrations of antibiotics, and grown shaking at 37°C until the OD_{450} was 0.5. IPTG was then added to a final concentration of 0.1mM and induction followed at 26°C for a further 2 hours. Bacteria were harvested by centrifugation and resuspended in 1% culture volume of resuspension buffer (50mM Hepes pH 7.2, 100mM NaCl, 6mM CHAPS, 1mM PMSF and 0.1mM DTT) and frozen at −20°C. Thawed bacteria were disrupted by sonication for 4×10 secs. at 80−90 W (using a Dawe soniprobe Type7532A) on ice prior to addition of DNase I (to 24μg/ml); RNase A (to 100μg/ml) and $MgCl_2$ (to 6mM). After 30 mins incubation at 4°C, NaCl was added to 1M final concentration followed by addition of polymin P to 0.2%. The resulting suspensions were centrifuged at 15K for 15 mins. in the Sorvall SS34 rotor. Supernatants were made up to 35% saturation with ammonium sulphate and the precipitated protein pellets dissolved in 0.2% original culture volume of resuspension buffer. These solutions were clarified by centrifugation and the supernatants containing the partially purified extracts stored at −20°C. A partially purified extract of the HSV-1 Vmw175 DNA binding domain peptide I9X (31) prepared by this method was kindly provided by K. E. Allen.

Polyacrylamide gel analysis of expressed proteins

E.coli BL21 bacterial extracts containing the induced viral proteins were analyzed by electrophoresis through a 12.5% SDS polyacrylamide gel using the Biorad miniprotean II kit and visualized by staining with Coomassie blue.

Preparation of probes for DNA binding analyses

End-labelled restriction fragment probes used for DNA binding analyses on the HSV-1 IE-3 promoter and the VZV gene 62 promoter regions were prepared as follows:- The UP6 probe, including the gene 62 mRNA start site at nucleotide −287 proposed by Felser (12) was isolated as a SalI-RsaI fragment, spanning nucleotides −409 to −188 of the gene 62 promoter, from the plasmid p140 (28). The BT probe, spanning the mRNA start site at nucleotide +1 located experimentally by McKee (28) was isolated as a XhoI-BglII fragment encompassing residues −130 to +57 of the gene 62 promoter from the plasmid p140BT, a derivative of p140 with a BglII site inserted at nucleotide +57 (28). The IE-3 probes contained a strong Vmw175 binding site (23) located at the cap site of the HSV-1 IE-3 gene and were isolated as either an AvaI-BamHI fragment (coordinates −18 to +27) or as an EcoRI-BamHI fragment (coordinates −108 to +27) from the plasmid pIE-3CAT (20). The probes were constructed by initially cutting the plasmids with either SalI, XhoI or BamHI respectively, end-labelling with Klenow and α^{32}P dATP, followed by digestion with the second enzyme as appropriate. The labelled fragments were eluted from acrylamide gels and purified by ethanol precipitation. A specific oligonucleotide probe (UP6 oligo) was synthesized corresponding to the region of the gene 62 promoter spanning the proposed mRNA start site at −287, by annealing complementary single stranded 35mer oligonucleotides carrying sequences derived from −311 to −283 of the gene 62 promoter flanked by non-gene 62 sequences giving rise to 5′ AvaI and 3′ BamHI sticky ends. Klenow end-filling, in the presence of α^{32}P dATP yielded the end-labelled UP6 oligo probe.

Comparative gel retardation analysis of the IE-3 consensus binding site containing single point mutations used probes prepared by the 'prime cut' method (32) taking measures to obtain sets of probes of equal specific activity. In each series of probe preparations, a wild type control was prepared in an identical manner at the same time.

DNA bending analysis of the gene 62 promoter sites used derivatives of the plasmid pBEND2 (33) which includes a tandemly repeated sequence (containing 17 different restriction sites) with unique XbaI and SalI cloning sites between the two repeats. Plasmids pBENDU and pBENDP were constructed by insertion of the gene 62 promoter UP6 oligo (including gene 62 bases −311 to −283) and an RsaI-RsaI gene 62 promoter fragment (−23 to +50) respectively into the SalI site of pBEND2 by blunt end ligation following Klenow end-filling of any 5′ overhangs. The direct repeats in these pBEND plasmids, contain amongst others, BglII, XhoI and BamHI sites; cutting with any of these enzymes produced DNA fragments of 184 bp from pBENDU and 218 bp from pBENDP. These fragments were Klenow end-labelled and isolated as before to produce two groups of three probes with 140k binding sites in circularly permuted positions with respect to their ends.

Gel retardation assays

Appropriate amounts of partially purified DNA binding domain peptides or nuclear extracts were incubated on ice with 0.1ng of end-labelled DNA fragment probes or freshly made 'prime cut' probes in buffer giving final concentrations of 10mM Tris.HCl pH8.0, 1mM EDTA, 100mM NaCl, 1μg polydI.polydC, 0.1% NP40 and 50μg/ml BSA. After incubation for 20 mins, 0.2 volumes of loading buffer (10mM Tris.HCl pH8.0, 1mM EDTA, 10mM mercaptoethanol, 0.1% CHAPS, 50% glycerol and 0.25mg/ml bromophenol blue) was added to each incubation mix. Samples were applied to a non-denaturing 4% polyacrylamide gel with 0.5×TBE running buffer and run at 150V at 4°C for 3 hrs. Complexes and unbound probe were detected by autoradiography of dried gels. Where appropriate, the extent of binding was determined by scintillation counting of dried gel slices corresponding to the positions of free and bound probe previously located by autoradiography. To allow for background values contributed by complexes with *E.coli* proteins (which were visually undetectable), dried gel slices from control lanes were also cut out and counted. These values, which were negligable, were subtracted from the sample counts, to allow calculation of the percentage of total probe bound.

DNase I footprinting assays

Site specific protection of DNA fragments from partial DNase I digestion by the 140k VT2 and Vmw175 I9X DNA binding domain peptides was determined by modification of the method of Galas and Schmitz (34). DNA binding reactions contained 4ng of target DNA labelled with ^{32}P at one 5′ end and suitable amounts of bacterial extracts, incubated in buffer giving final concentrations of 10mM Tris.HCl pH 7.5, 1mM EDTA, 10mM mercaptoethanol, 0.1% CHAPS and 100mM NaCl. After a 20 minute incubation at 22°C, $MgCl_2$ (to 10mM), $CaCl_2$ (to 2mM) and 0.0625 units of DNase I were added, mixed and incubated for a further 60 seconds, after which time 20μg of proteinase K in 100μl of a buffer containing 1% SDS, 100μg/ml tRNA, 200mM NaCl, 20mM EDTA was added and the reaction incubated at 50°C for a further 20 mins. The DNA was then

extracted by phenol, chloroform and ethanol precipitation and resuspended in formamide dye mix (1mg/ml xylene cyanol, 1mg/ml bromophenol blue and 10mM EDTA in deionized formamide). In addition, a G+A Maxam-Gilbert sequencing reaction (35) was carried out on 4ng of the particular end-labelled probe used in each DNase I footprinting experiment to provide a sequencing ladder for orientation of the footprint. Samples were applied to 8% polyacrylamide/8M urea sequencing gels for BT and UP6 probes and 12% polyacrylamide/8M urea gels for the shorter HSV-1 IE-3 AvaI-BamHI probe, and electrophoresed at 40W for 2–3 hrs. as required. Gels were dried prior to visualization of DNase I protection patterns by autoradiography.

RESULTS

Cloning and expression of the DNA binding domain of 140k in a T7 expression vector and its subsequent partial purification

Gene 62, encoding the 140k protein, lies in the left repeat bounding the short unique segment of the VZV genome (Figure 1); an identical copy (gene 71) lies in the opposite orientation in the terminal short repeat. The DNA binding domain of the homologous protein of HSV-1, Vmw175, has been expressed in isolation and found to have a similar binding specificity to that of the intact protein (21,25,31). Our intention was to express the equivalent region of the homologous VZV IE protein 140k, encompassing its DNA binding domain, in order to study its DNA binding characteristics for comparison with those of the HSV-1 Vmw175 DNA binding domain. Wu and Wilcox (27) had previously expressed in *E.coli* the 140k codons 417 to 647, preceeded by 323 codons derived from the trpE ORF, and had found that this fusion peptide bound DNA in a sequence-specific manner. We chose to express a similar region of 140k as a non-fusion polypeptide to eliminate the possible influence of a large stretch of non-homologous *E.coli* coding region on the properties of the fusion protein. Our non-fusion polypeptide spanned VZV 140k codons 417 to 646 and mainly consisted of the highly conserved region 2, designated on the basis of homology between the HSV-1 Vmw175 and the VZV 140k proteins (18). The C-terminal portion of region 1, poorly conserved in the related IE proteins of the α-herpesviruses, was also included in our expressed peptide; the role played by these additional residues is not fully understood but deletion analysis on the 140k DNA binding domain found them to be necessary for the production of a clear DNase I footprint but not for binding in gel retardation assays (see below).

The DNA binding domain of 140k was cloned into the T7 expression vector p585T7a, as described in detail in the materials and methods section. The resulting plasmids p585T7aVT2 and p585T7aVT2X both contained the gene 62 coding region for 140k residues 417–646 inclusive, differing from each other only by the extent of vector coding sequences to the 3′ of this gene 62 region. The expressed polypeptides will be referred to as VT2 and VT2X respectively. Both of these 140k DNA binding domain peptides were expressed to high levels and were clearly visible on SDS-PAGE gels stained with Coomassie blue (Figure 2). The VT2X version of VT2 was constructed due to the finding that

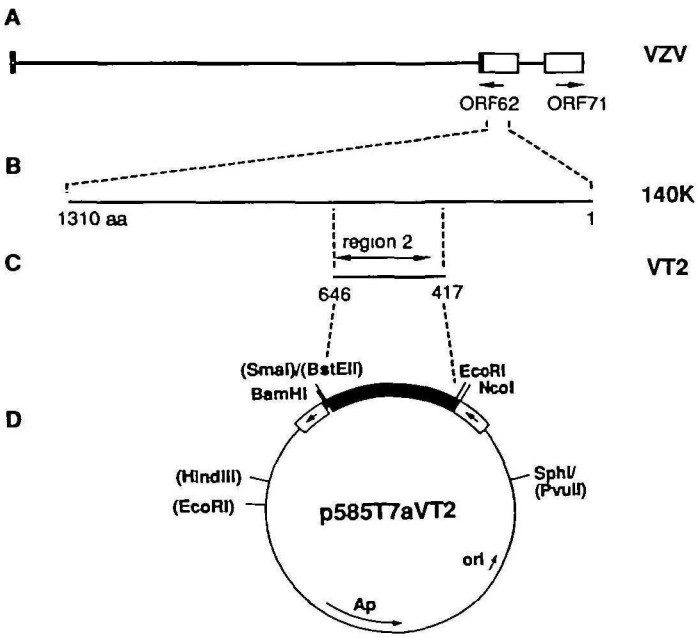

Figure 1. A. The structure of the VZV genome with the repeated sequences shown as boxes and the positions of the ORF 62 and ORF 71 marked in the short repeats. B. Expansion of the 140k coding region (1310 amino acid residues in length). C. The location of the region of 140k encompassing its DNA binding domain, this VT2 region includes 51 residues from the C-terminal portion of non-conserved region 1, the whole of region 2 (codons 468–641) and 5 residues from region 3. D. The 140k DNA binding domain inserted into the T7 expression vector p585T7a to give plasmid p585T7aVT2. Only positions of restriction sites altered during the construction of the p585T7a vector and also sites relevant to the subcloning of the 140k VT2 region are indicated. The location of the T7 transcriptional initiation and termination signals are marked by open boxes containing arrows to indicate the direction of expression. The black box represents the NcoI-EcoRI oligonucleotide adaptor inserted to maintain the 140k reading frame and to facilitate the insertion of the EcoRI-BstEII gene 62 fragment from plasmid pV17 into the vector p585T7a. The shaded box represents the 140k coding region (codons 417–646).

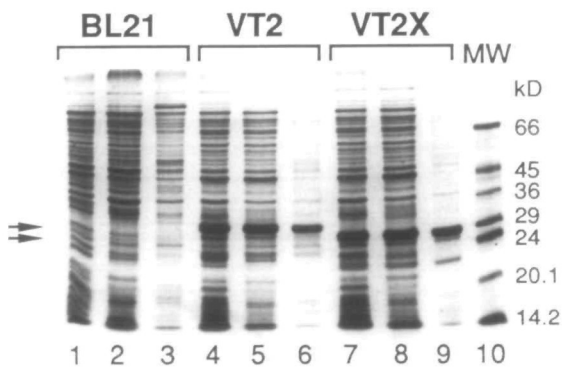

Figure 2. Expression of the 140k DNA binding domain in *E.coli*. Bacteria were grown, expression from the T7 plasmids induced and crude extracts were prepared by ammonium sulphate precipitation and analyzed on 12.5% SDS PAGE. BL21 is an extract prepared from induced bacteria without an expression plasmid, VT2 and VT2X indicate extracts prepared from bacteria carrying plasmids p585T7aVT2 and p585T7aVT2X respectively. Lanes 1,4,7—lysed bacteria after harvesting; lanes 2,5,8—post polymin P precipitation supernatants; lanes 3,6,9—crude extracts after 35% ammonium sulphate precipitation. The amount of extract in each lane corresponds to that produced from 3ml of induced bacterial culture. Lane 10—molecular weight standards; BSA, 66kD; albumin, 45kD; glutataldehyde-3-phosphate dehydrogenase, 35kD; trypsinogen, 24kD; trypsin inhibitor, 20.1kD; lactalbumin, 14.2 kD. The positions of VT2 (28.5kD) and VT2X (26.4kD) peptides are marked by arrows.

'X' versions of the HSV-1 Vmw175 DNA binding domain peptides were generally more stable than the non-X versions (31). The VT2X polypeptide was expressed at slightly higher levels and had an increased DNA binding affinity as compared to the VT2 version (data not shown); otherwise these two proteins could be considered identical. Partially purified extracts of bacterially expressed VT2 and VT2X were obtained by ammonium sulphate precipitation (Figure 2) and were used for all subsequent DNA binding analyses.

The 140k DNA binding domain specifically recognizes multiple gene 62 promoter sequences in DNase I footprinting assays

Initial experiments using the VT2X peptide with the UP6 probe (described in materials and methods) indicated the formation of defined DNA:protein complexes. With increasing amounts of protein, higher order complexes containing more than one VT2X peptide were observed (results not shown, but see Figure 5).

We investigated the interaction of VT2 on a number of different probes by DNase I footprinting. Typical results (Figure 4) indicated that VT2 bound (with apparently similar affinity) to several sites within the gene 62 promoter region (summarized in Figure 3), and that many of these sites contained sequences similar to the HSV-1 Vmw175 binding site consensus (Figure 3).

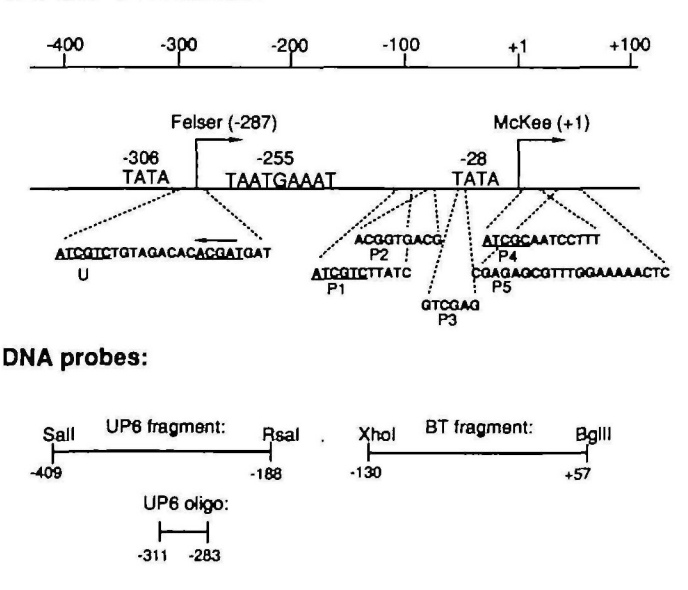

Figure 3. Schematic diagram of the gene 62 promoter region. Numbers on the top scale indicate nucleotide positions corresponding to the gene 62 promoter. The locations of both proposed mRNA start sites are shown by bent arrows. A TATA box homology TTTTAA is centered at nucleotide −28. A sequence at −261 to −249 contains both octamer and TAATGARAT motifs (28). The proposed start site at −287 also has a TATA box homology (TATAT) centered at nucleotide −306. Expansions of the gene 62 promoter sequences (coding strand) specifically protected from DNase I digestion by the 140k DNA binding domain peptide VT2 are also given. These sequences are labelled U, P1 to P5 and obvious homologies to the motif found in the 5′ portion of the HSV-1 Vmw175 binding consensus are underlined, the arrow indicating a motif in the opposite orientation on the non-coding strand. The bottom section shows the relative positions of the probe fragments derived from the gene 62 promoter and the restriction sites used for their isolation. In the case of the UP6 oligo, the numbers refer to the region of the gene 62 promoter included in the oligonucleotide probe sequence.

Characteristically these sites were flanked by regions of DNase I hypersensitivity (Figure 4).

The 140k DNA binding domain recognizes the HSV-1 IE-3 promoter Vmw175 binding site in gel retardation and footprinting analyses

Several of the VT2 binding sites, as identified by the DNase I footprinting analysis on the gene 62 promoter, showed sequence homology to the HSV-1 Vmw175 DNA binding consensus AT-CGTnnnnnYSG (21). This had been noted previously (27) but in view of the observation that VZV 140k did not repress the HSV-1 promoter (17) we decided to investigate the relationship

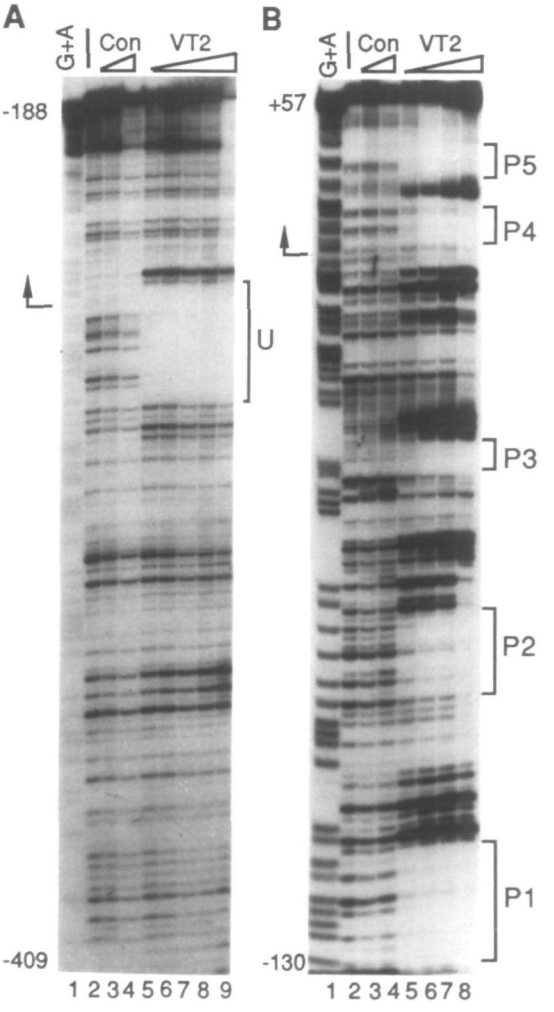

Figure 4. DNase I footprints on the VZV gene 62 promoter. DNase I footprinting assays were performed with 5′ end-labelled DNA fragments and bacterial extracts. Target DNAs derived from the gene 62 promoter region were (A) UP6 fragment (bp −409 to −188) and (B) BT fragment (bp −120 to +57), in both cases the non-coding strand was labelled. Arrows indicate the proposed transcriptional initiation sites at nucleotide −287 in (A) and at +1 in (B). Brackets representing regions of protection from DNase I digestion are labelled U (−302 to −280), P1(−107 to −97), P2 (−81 to −74), P3 (−52 to −47), P4 (+6 to +18) and P5 (+27 to +46). Tracks labelled G+A, −and con contain Maxam and Gilbert sequencing reaction, no extract and induced bacterial extract carrying the p585T7a vector respectively. Tracks labelled VT2 contain bacterial VT2 extract. Triangles represent progressively increasing amounts of extract in the lanes covered by the triangle, the amounts of control extract corresponding to the same range of amounts of the VT2 extract used.

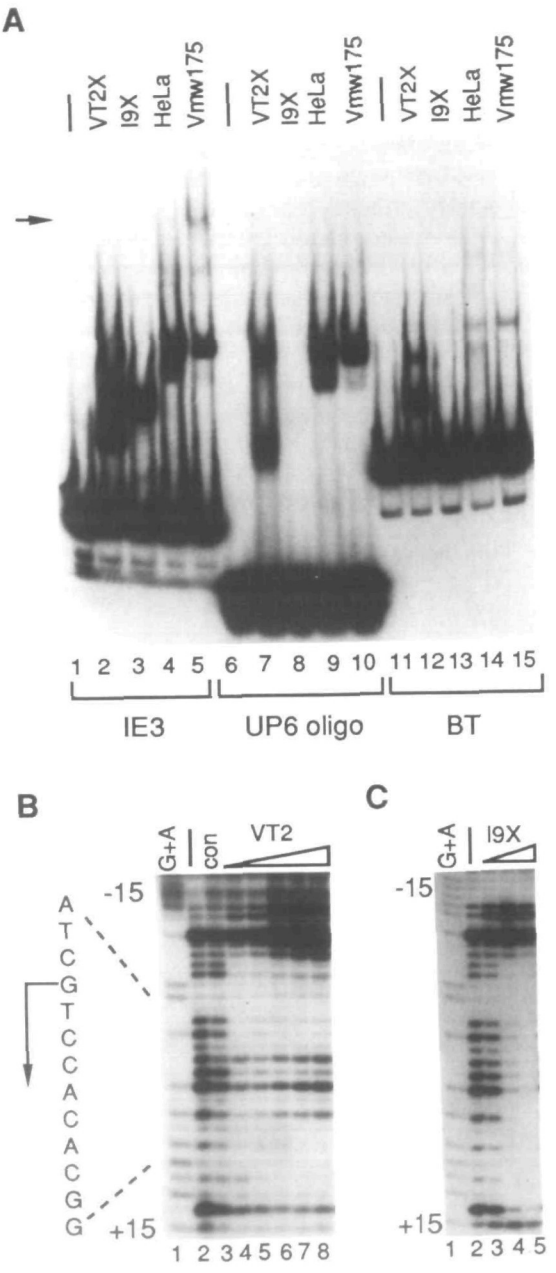

Figure 5. A. Gel retardation analysis of HSV-1 IE-3 and VZV gene 62 promoter regions. Target DNAs; lanes 1–5 contain HSV-1 IE-3 promoter EcoRI-BamHI fragment probe (−108 to +27), lanes 6–10 contain UP6 oligo probe including gene 62 promoter sequences from −311 to −283, lanes 11–15 contain the gene 62 promoter XhoI-BglII fragment BT probe (−130 to +57). These target DNAs were incubated with approximately equivalent amounts of VZV VT2X or HSV-1 I9X bacterial extracts, or equivalent amounts of HeLa nuclear extracts with or without intact Vmw175. Prolonged autoradiographic exposure of the gel (as shown here) was necessary to visualize the complex between the HSV-1 IE-3 promoter and intact Vmw175 (lane 5) marked by an arrow to the left hand side of panel A. Panels B. and C. show DNase I footprinting analyses of the HSV-1 IE-3 promoter AvaI-BamHI fragment (−18 to +27), but only nucleotides −15 to +15 are shown. In both panels; lane 1 contains a G+A Maxam and Gilbert sequencing reaction and lane 2 contains no extract. In B.; lane 3 contains an extract from bacteria containing plasmid p585T7a; lanes 4–8 contain increasing amounts of bacterial VZV VT2 extract. In C.; lanes 3–5 contain increasing amounts of bacterial HSV-1 I9X extract. An expansion of the sequence of the IE-3 promoter Vmw175 consensus binding site is shown and the direction and position of transcription initiation is marked by the arrow.

between VT2 and Vmw175 binding sites in detail. Firstly we confirmed that the VZV DNA binding domain could interact with the Vmw175 binding site in the IE-3 promoter. Incubation of the VT2X peptide with an IE-3 probe (coordinates −108 to +27) gave specific protein:DNA complexes (Figure 5A, lane 2) and again additional multiple complexes arose on titration of the VT2X peptide (data not shown) as noted earlier with the gene 62 promoter probes. Lesser exposures of the gel in Figure 5A show three such multiple VT2X complexes on the IE-3 probe (lane 2).

A high-resolution DNase I footprinting analysis on the AvaI-BamHI HSV-1 IE-3 fragment probe (−18 to +27) provided verification that the 140k DNA binding domain specifically recognized the HSV-1 IE-3 Vmw175 consensus binding site. The VT2 peptide protected, albeit faintly, both halves of this bipartite Vmw175 consensus site (21) from DNase I digestion (Figure 5B). DNase I hypersensitivity was again induced at the edges of the protected sequences as the amount of VT2 extract present increased. The footprint produced at this site by the VZV 140k VT2 DNA binding domain differed in several respects to that produced by protein I9X, the corresponding domain of Vmw175 (Figure 5C). VT2 induced greater DNase I hypersensitivity at the 5′ side of the binding site but less complete protection within

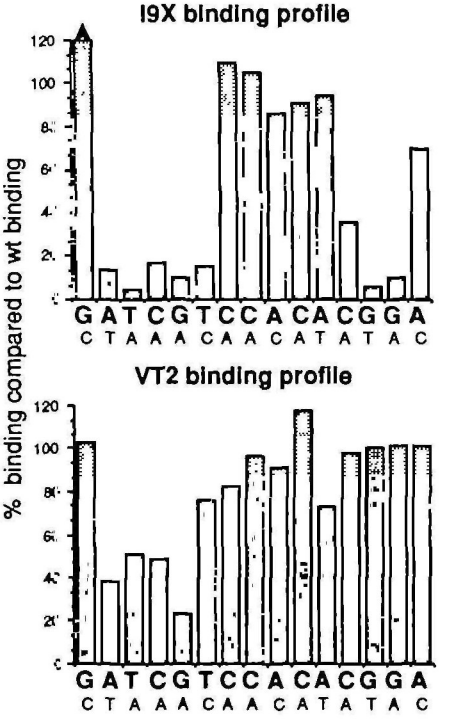

Figure 6. Comparison of the effect of point mutations in the HSV-1 IE-3 consensus binding site on the binding efficiencies of I9X and VT2. The sequence in the region of the IE-3 Vmw175 binding site is shown in large type at the bottom of both panels. The vertical bars above each base represent the percentage efficiency of binding when that base was mutated to the one shown in smaller type below (compared to binding efficiency to the wild type probe). Data used to produce the I9X binding profile was abstracted from reference 21. Analysis of VT2 binding efficiency was carried out with sets of mutagenized probe fragments produced on two separate occasions and gave highly reproducible results. Values used to produce the VT2 binding profile represent averages of the results provided by these two analyses.

the binding site itself. Strikingly VT2 induced hypersensitivity in the central section of the binding site while I9X completely protected this region. Therefore although both proteins bind to the same site, the details of their structural interactions must differ.

The 140k DNA binding domain exhibits reduced binding specificity for an HSV-1 Vmw175 consensus binding site, as compared to the corresponding domain of Vmw175

Having established that the 140k DNA binding domain recognized the HSV-1 IE-3 promoter binding site, we decided to compare the binding specificities of the HSV-1 Vmw175 and VZV 140k proteins. Our intention was to identify differences in the binding specificities of these two homologous IE proteins that might account for their opposing functional effects on the IE-3 promoter seen in transfection assays (17). An extensive mutagenesis of the IE-3 binding site had been performed previously for an analysis of the sequence requirements for Vmw175 binding (21). We utilized the progeny M13 phage resulting from this study to isolate 'prime cut' AvaI-BamHI IE-3 fragment (−18 to +27) probes, containing single point mutations at all positions throughout this IE-3 consensus binding site. The probes were used in gel retardation experiments with the VT2 bacterial extract to determine the VT2 binding specificity for the IE-3 binding site. The percentage efficiency of VT2 binding to each mutant probe was determined with respect to binding to the wild-type probe. The results are summarized in histogram form in the bottom section of Figure 6 with the previously determined binding profile for the Vmw175 DNA binding domain peptide I9X (21,31) shown above. It is apparent that the 140k DNA binding domain has an overall reduced binding specificity for this IE-3 consensus binding site compared to that of the Vmw175 DNA binding domain and appears to be virtually insensitive to point mutations in the 3′ portion of the bipartite sequence.

Both intact HSV-1 Vmw175 and its isolated DNA binding domain have very low affinities for the VZV gene 62 promoter VT2 binding sites

We had demonstrated that the 140k DNA binding domain specifically recognized the HSV-1 IE-3 promoter binding site and were interested to determine whether the HSV-1 Vmw175 DNA binding domain would interact with the 140k binding sites identified in the VZV gene 62 promoter. Approximately equivalent amounts of VT2X and the corresponding Vmw175 DNA binding domain peptide I9X (31) were incubated with IE-3 promoter and gene 62 promoter probes and the resultant complexes analyzed by gel retardation assays. We found that complexes specific to I9X were undetectable with the UP6 oligo probe (Figure 5A, lane 8) which spans the gene 62 promoter U site (strongly protected by VT2 in DNase I footprinting). I9X incubation with the BT probe, which includes the 140k binding sites P1−P5, also gave no obvious specific complexes (Figure 5A, lane 13). On repetition of this analysis with greater amounts of I9X extract, faint protein:DNA complexes were detected with the gene 62 promoter BT probe (data not shown). To verify that the binding results obtained for I9X reflect the situation with intact Vmw175, nuclear extracts from HeLa cells, transfected either with or without plasmid p175 (30) were also incubated with the same probes as above. The only protein:DNA complex specific to intact Vmw175 produced in this gel retardation analysis was with the HSV-1 IE-3 probe (seen in lane 5 of Figure 5A, marked by the horizontal arrow), the complexes obtained with the UP6 oligo and BT gene 62 promoter probes being identical for nuclear extracts with or without Vmw175 (Figure 5A, lanes 10 and 9, 15 and 14 respectively). We conclude that intact HSV-1 Vmw175 and its isolated DNA binding domain both have greatly reduced affinities for the VT2 binding sites in the VZV gene 62 promoter, compared to the binding observed with the VZV 140k DNA binding domain. So although several of the VT2 binding sites in the gene 62 promoter show sequence similarity to the Vmw175 binding consensus, the degree of homology of the sites is insufficient to allow recognition of these gene 62 promoter sites by the comparatively specific Vmw175 protein. These results are in agreement with those obtained from the above specificity comparison between VZV VT2 and HSV-1 I9X DNA binding domain peptides, which found VT2 to be generally less sequence-specific than the Vmw175 DNA binding domain. In addition, HSV-1 I9X protection of the strong VT2 binding site U in the VZV gene 62 promoter was undetectable

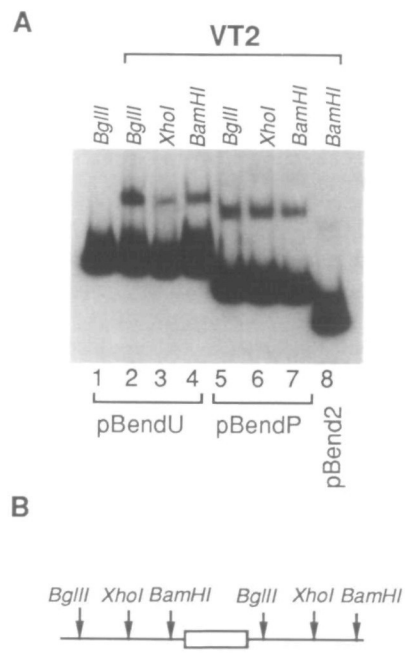

Figure 7. The circular permutation gel retardation assay with the VT2 peptide. A. Circular permutation gel retardation assay with the gene 62 promoter U and P4/P5 binding sites. Lane 1 contains free probe while lane 8 contains probe derived from the plasmid pBEND2 by BamHI digestion. Probes in lanes 1,2,3 and 4 (which include site U) were derived from the plasmid pBENDU by cutting with the relevant enzymes indicated above each lane. Similarly for probes in lanes 5,6 and 7 (which include sites P4 and P5) were derived from plasmid pBENDP. Incubation mixes for lanes 2−8 contained sufficient VT2 extract to produce only primary complexes and the amount of non-specific inhibitor (polydI:polydC) was increased to 4μg per reaction to reduce the extent of non-specific VT2 binding to the non-gene 62 promoter derived probe regions. Non-specific DNA binding still occurred, represented by the faint complex seen in lane 8 containing the pBEND2 probe with no introduced VT2 binding site, but this non-specific binding complex is a minor species compared to the specific primary complexes apparent in lanes 2−7. B. Schematic showing a binding site containing fragment (depicted by the box) inserted between the pBEND2 derived tandemly repeated sequence. BglII, XhoI and BamHI restriction sites are at equivalent positions in each of the repeats as shown. Single enzyme digestions yield fragments of identical lengths but with the position of the binding site circularly permuted with respect to the ends of the probes.

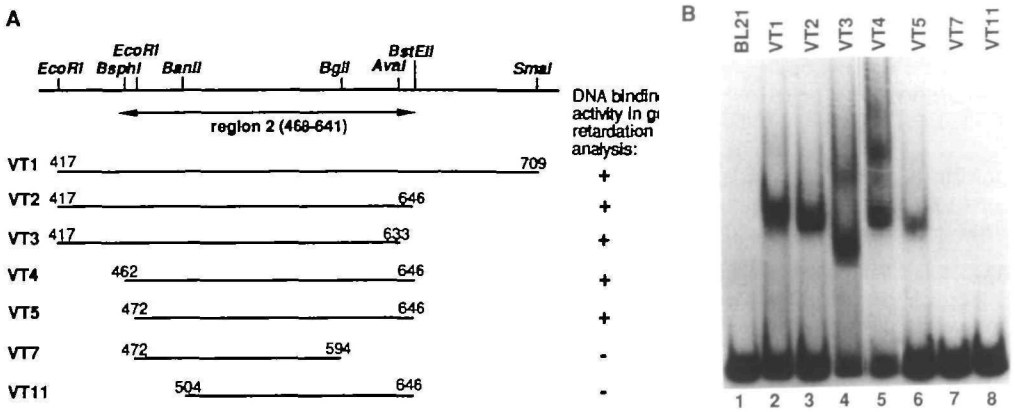

Figure 8. A. Schematic diagram of the deletion constructs of the VZV 140k DNA binding domain. The expressed regions of the 140k protein (labelled VT1−VT5, VT7 and VT11) are indicated by horizontal lines. The numbers of the 140k residues at the amino- and carboxy-terminal ends of each deletion polypeptide are given and the corresponding restriction sites used for the isolation and cloning of gene 62 sequences into the T7 expression vectors are shown above. The gene 62 fragment used in the construction of the plasmid expressing VT5 was isolated from plasmid pV18 (39) which is a derivative of p140 (28) with a 12bp EcoRI linker oligonucleotide inserted at codon 472. The remaining gene 62 fragments were all isolated from plasmid pV17 (refer to materials and methods). The ability of each deletion polypeptide to bind to DNA (+) or not (−) in gel retardation analyses is summarized on the right hand side of panel A. **B.** Gel retardation experiment with the 140k DNA binding domain deletion polypeptides. Partially purified bacterial extracts of each deletion polypeptide were incubated with the HSV-1 IE3 promoter AvaI-BamHI fragment probe and resultant complexes analyzed by electrophoresis. Similar results were obtained with VZV gene 62 promoter fragment probes. The particular deletion polypeptide extract present in each incubation is indicated for lanes 2−8, BL21 indicates an extract from bacteria carrying plasmid p585T7a.

using DNase I footprinting analysis of the UP6 fragment probe (data not shown).

Binding of the 140k DNA binding domain does not induce DNA bending at its gene 62 promoter binding sites

The isolated DNA binding domain of HSV-1 Vmw175 causes a significant DNA bend in the immediate vicinity of the Vmw175 binding sequence spanning the HSV-1 IE-3 mRNA start site (26). This may play a role in the autoregulatory function of Vmw175. Since we had found that the 140k DNA binding domain recognizes sites bearing similarity to the Vmw175 consensus binding sequence, and moreover, specifically recognizes this IE-3 promoter binding site, it was of interest to determine whether VT2 binding would induce a similar conformational change on the DNA at its binding sites in the vicinities of both proposed gene 62 mRNA start sites. The method used to detect DNA bending depends on the principle that a bend near the end of a restriction fragment causes a less pronounced reduction in electrophoretic mobility as compared to a bend in the middle. In order to test for VT2 induced DNA bending, we cloned regions of the gene 62 promoter, spanning the U site and also the P4/P5 140k binding sites (identified by the DNase I footprinting analysis) into the pBEND2 plasmid (33). Probe fragments derived from the resultant pBENDU and pBENDP plasmids, by cutting with either BglII, XhoI or BamHI, contained respectively a single or pair of VT2 binding sites at circularly permuted positions within each set of three probes (Figure 7B). The VT2 DNA binding domain peptide VT2 was incubated with each of the probes and the resulting complexes separated on polyacrylamide gels. An example of a typical bending analysis is shown in Figure 7A; there is no significant difference in mobility of the primary protein:DNA complex within each set of three probes derived from either the pBENDU or pBENDP plasmids. Thus the 140k DNA binding domain does not appear to induce DNA bending at the VT2 binding sites in the vicinities of the proposed gene 62 mRNA start sites.

The DNA binding activity of VZV 140k resides within residues 472 to 633 as determined by gel retardation analysis

VZV 140k DNA binding domain deletion polypeptides were expressed to high levels in *E.coli* and partially purified as described for the VT2 polypeptide. Details of the expressed regions are given in Figure 8A. The DNA binding ability of each deletion polypeptide was assayed by gel retardation analysis, an example of which is shown in Figure 8B. Polypeptides VT1 to VT5 gave well defined protein:DNA complexes while VT7 (residues 472−594) and VT11 (residues 504−646) failed to bind to the DNA probe (Figure 8B, lanes 7 and 8 respectively) even at greatly increased protein concentrations (data not shown). From this deletion analysis we conclude that the minimal DNA binding domain of the 140k protein lies within residues 472 to 633. The VT2 peptide was selected for all of the above DNA binding analyses for several reasons. The longer VT1 peptide was less stable than VT2 and tended to degrade in the bacterial extracts. The shorter peptides VT4 and VT5 gave good DNA binding in gel retardation assays, but, for reasons unknown, failed to yield DNase I footprints at the VT2 binding sites described above. Despite this lack of footprinting, the specificity of binding of the shorter VT4 peptide was very similar to that shown in Figure 6B using VT2 (data not shown). Although the VT3 peptide bound to DNA in both types of assays, it is interesting that the mobility of the VT3 complex is greater than expected (Figure 8B, lane 4). It is possible that the deletion is encroaching upon an important part of the protein and altering the conformation of the complex.

DISCUSSION

The results in this paper demonstrate that a portion of the VZV 140k protein comprising the C-terminal portion of region 1 and all of the conserved region 2 can be expressed in isolation in *E.coli* as a non-fusion DNA binding domain peptide that interacts with multiple sequences in the VZV gene 62 promoter. Several

of these sequences are similar to the HSV-1 Vmw175 binding consensus, although numerous differences in binding characteristics between the homologous VZV 140k and HSV-1 Vmw175 DNA binding domain peptides have been identified. The possible functional implications of these observations are considered below.

We chose to utilize the isolated DNA binding domain of the VZV 140k protein for these analyses due to the difficulties encountered in a previous attempt to detect sequence-specific DNA binding by the intact 140k polypeptide (39). The sequence-specific DNA binding function of HSV-1 Vmw175 resides in a discrete domain of the polypeptide comprising mainly the highly conserved region 2 sequences. This Vmw175 domain, when expressed in bacteria or liberated from the intact protein by proteolytic cleavage, is able to bind DNA with a very similar affinity and specificity to the intact polypeptide (25,21,24). Therefore it is reasonable to assume that the binding properties of the 140k DNA binding domain presented here reflect those of the full 140k polypeptide.

Autoregulation of the HSV-1 IE-3 gene requires (at least in transfection assays) interaction of Vmw175 with a specific binding site in the IE-3 promoter (36). Similarly the 140k DNA binding domain specifically recognizes its own gene 62 promoter and by analogy to the situation with Vmw175, this interaction is implicated in the autoregulatory function of 140k. The ability to bind multiple sites in the gene 62 promoter had been recognized previously by Wu and Wilcox (27) using a 140k region 2 domain fusion protein; our analyses utilizing a non-fusion 140k DNA binding domain polypeptide (VT2) confirm and extend this observation. The VT2 peptide exhibits slightly differing affinities for the numerous binding sites in the gene 62 promoter, but preferential binding to any single site was not apparent.

Binding to multiple sites is unlikely to be a feature specific to the autoregulatory function of 140k, since DNase I footprinting analysis of the HSV-1 glycoprotein gD promoter, which is activated by 140k in transfection assays (15), identifies multiple sites of VT2 protection and extensive DNase I hypersensitivity (data not shown). In addition, the homologous IE protein of PRV (IE180) binds multiple sequences with limited homology in the AdML and hsp70 promoters and such binding is necessary for the activation of these promoters by the PRV IE180 protein (37).

No obvious 140k binding consensus could be derived from the gene 62 promoter binding sites, although several of the more strongly protected sequences contain homologies to the 5′ portion of the Vmw175 binding consensus (21, 22). Interestingly two such sites are located in the immediate vicinities of both the transcription initiation site at +1 and the proposed transcription initiation site further upstream at −287. A thorough mutational analysis of the gene 62 promoter would be necessary to determine whether these, or any other specific binding sites, are required for the downregulation of this promoter by the 140k protein.

Our examination of the specificity of VT2 for a Vmw175 consensus binding site found that sequences in the 3′ portion of the consensus site were unimportant for VT2 binding. It is possible that the VT2 binding sites may have their own distinct 3′ sequence, but none is apparent for the sites identified in this study. The results presented here show the 140k DNA binding domain has a lower DNA binding specificity compared to the equivalent domain of Vmw175. This is reflected in the high number and the variety of the sequences protected by VT2 in the gene 62 (Figure 3) and HSV-1 gD promoters and also the high level of non-specific binding to the pBEND2 probe (Figure 7A, lane 8). This low specificity of DNA binding might perhaps explain the promiscuous transactivating phenotype of 140k in transfection assays (15,16). It can be predicted from the low binding specificity of the 140k DNA binding domain, that the intact 140k protein would have a low promoter selectivity; 140k could perhaps function by activating sub-optimally utilized RNA polymerase II promoters in a similar manner to that previously suggested for the homologous PRV IE180 protein (38). The 140k protein is also a more potent transactivator than Vmw175 in transfection assays (15,16). This is not merely a function of the relative strengths of the HSV-1 IE-3 and VZV gene 62 promoters (39) but could be attributable to either the relative strengths of their activating domains (unknown at present) or alternatively multiple 140k interactions with the promoter may have an additive effect, enhancing the efficiency of activation.

Our results suggest that DNA binding to a sequence over the transcription initiation site is not sufficient for autoregulation as protein VT2 specifically interacts with the HSV-1 IE-3 consensus site yet evidence from transfection assays (17) and also the recombinant HSV-140 virus (13) suggests that intact 140k fails to repress this IE-3 promoter. In addition, although Vmw175 is equally as effective as 140k in the downregulation of the gene 62 promoter in transfection assays (17), intact Vmw175 and also its isolated DNA binding domain displayed greatly reduced affinities for the binding sites therein, compared to the 140k DNA binding domain. Slight differences between the sequences of the specific binding sites found in the region of the IE-3 and gene 62 mRNA start sites may account for this functional inconsistency. Alternatively the precise positioning of the IE-3 site with respect to the mRNA start site may not be correct for downregulation of this promoter by the 140k protein, as a close correlation has been observed between Vmw175 mediated autorepression and the stereo-specific or distance-dependent placement of the IE-3 binding site and the TATA box (discussion of reference 40). It seems that the mechanism of autoregulation of the gene 62 promoter is more complicated than DNA binding alone and is likely to involve interactions with other proteins.

Of possible functional significance are the alterations of DNA conformation induced by binding of the VT2 peptide, made apparent by the strong sites of DNase I hypersensitivity in the gene 62 promoter (Figure 4). The purified DNA binding domain of Vmw175 significantly bends the DNA at its binding sites (26), whereas VT2 induced bending was not detected in a corresponding analysis. This lack of DNA bending at individual VT2 binding sites and also the extensive nature of the hypersensitivity pattern suggest that the conformational effects may extend along the whole promoter, induced by multiple VT2 peptides binding to numerous sites. If this transpires to be the case for binding of the intact 140k protein during VZV infection, numerous possible functional implications of such a broad-ranging conformational effect can be envisaged, including modification of the interactions of transcription factors with DNA or with other proteins, or clearing the promoter of non-specific DNA binding proteins.

Despite the equivalent roles played by the HSV-1 Vmw175 and VZV 140k proteins in their respective viruses and the high level of sequence homology between their DNA binding domains, numerous differences in their DNA binding characteristics are apparent and are likely to have an important bearing on their activities during infection.

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REFERENCES

1. Honess,R. and Roizman,B. (1974) J. Virol., **14**, 8–19.
2. Clements,J.B., Watson,R.J. and Wilkie,N.M. (1977) Cell, , 275–285.
3. Everett,R.D. (1987) Anticancer Research, **7**, 589–604.
4. Wagner,E.K. (1985) In: The Herpesviruses. Ed. Roizman,B. Plenum Press, New York/London. **3**, 45–104.
5. Roizman,B. and Sears,A.E. (1990) In: Virology, 2nd edition. Eds Fields,B.N., Knipe,D.M. et al., Raven Press, New York. **2**, 1795–1841.
6. Preston,C.M. (1979) J.Virol., **29**, 275–284.
7. DeLuca,N.A., McCarthy,A.M. and Schaffer,P.A. (1985) J. Virol., **56**, 558–570.
8. Davison,A.J. and Scott,J.E. (1986) J. Gen. Virol., **67**, 1759–1816.
9. McGeoch,D.J., Dalrymple,M.A., Davison,A.J., Dolan,A., Frame,M.C., McNab,D. Perry,L.J., Scott,J.E. and Taylor,P. (1988) J. Gen. Virol., **69**, 1531–1574.
10. Shiraki,K. and Hyman,R.W. (1987) Virology, **156**, 423–426.
11. Felser,J.M., Straus,S.E. and Ostrove,J.M. (1987) J. Virol., **61**, 225–228.
12. Felser,J.M., Kinchington,P.R., Inchauspe,G., Straus,S.E. and Ostrove,J.M. (1988) J. Virol., **62**, 2076–2082.
13. Disney,G.H. and Everett,R.D. (1990) J. Gen. Virol., **71**, 2681–2689.
14. Everett,R.D. and Dunlop,M. (1984) Nucleic Acids Res., **12**, 5969–5978.
15. Everett,R.D. (1984) EMBO J., **3**, 3135–3141.
16. Inchauspe,G. and Ostrove,J.M. (1989) Virology, **173**, 710–714.
17. Disney,G.H., McKee,T.A., Preston,C.M. and Everett,R.D. (1990) J. Gen. Virol., **71**, 2999–3003.
18. McGeoch,D.J., Dolan,A., Donald,S. and Brauer,D.H.K. (1986) Nucleic Acids Res., **14**, 1727–1764.
19. DeLuca,N.A. and Schaffer,P. (1988) J. Virol., **62**, 732–743.
20. Paterson,T. and Everett,R.D. (1988) Virology, **166**, 186–196.
21. Everett,R.D., Elliott,M., Hope,G. and Orr,A. (1991) Nucleic Acids Res., **19**, 4901-4908.
22. DiDonato,J.A., Spitzner,J.R. and Muller,M.T. (1991) J. Mol. Biol., **219**, 451–470.
23. Muller,M. (1987) J. Virol., **61**, 858–865.
24. Everett,R.D., Paterson,T. and Elliott,M. (1990) Nucleic Acids Res., **18**, 4579–4585.
25. Wu.C.L. and Wilcox,K.W. (1990) Nucleic Acids Res., **18**, 531–538.
26. Everett,R.D., DiDonato,J., Elliott,M. and Muller,M. (1992) Nucleic Acids Res., **20**, 1229–1233.
27. Wu,C.L. and Wilcox,K.W. (1991) J.Virol., **65**, 1149–1159.
28. McKee,T.A., Disney,G.H., Everett,R.D. and Preston,C.M. (1990) J. Gen. Virol., **71**, 897–906.
29. Studier,F.W., Rosenberg,A.H., Dunn,J.J. and Dubendorff,J.W. (1990) Methods in Enzymology, **185**, 60–89.
30. Paterson,T. and Everett,R.D. (1988) Nucleic Acids Res., **16**, 11005–11025.
31. Pizer,L.I., Everett,R.D., Tedder,D.G., Elliott,M. and Litman,B. (1991) Nucleic Acids Res., **19**, 477–483.
32. Biggin,M., Farrell,P.J. and Barrell,B.G. (1984) EMBO J., **3**, 1083–1090.
33. Zweib,C., Kim,J. and Adhya,S. (1989) Genes Dev., **3**, 606–611.
34. Galas,D. and Schmitz,A. (1978) Nucleic Acids Res., **9**, 3047–3060.
35. Maxam,A.M. and Gilbert,W. (1980) Methods in Enzymology, **65**, 499–560.
36. Roberts,M.S., Boundy,A., O'Hare,P., Pizzorno,M.C., Ciufo,D.M. and Hayward,G.S. (1988) J. Virol., **62**, 4307–4320.
37. Cromlish,W.A., Abmayr,S.M., Workman,J.L., Horikoshi,M. and Roeder,R.G. (1989) J. Virol., **63**, 1869–1876.
38. Thali,M., Rusconi,S. and Schaffner,W. (1990) J. Mol. Biol., **215**, 301–311.
39. Disney,G.H. (1990) Ph.D. Thesis, University of Glasgow.
40. DiDonato,J.A. and Muller,M.T. (1989) J. Virol., **63**, 3737–3747.